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KEY FACTORS INFLUENCING JUVENILE QUALITY IN MARICULTURE: A REVIEW

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Key words: cortisol, essential fatty acids, fish larvae, growth, juvenile quality, metamorphosis, pigmentation, thyroxine

Abstract

Environmental (temperature, salinity) and nutritional (DHA, EPA, ArA, vitamin A, phospholipids, iodine) factors during larvae rearing largely dictate the successful transformation of larvae to juveniles during metamorphosis which, in turn, determines juvenile quality. Studies on Atlantic halibut, turbot and Japanese flounder report higher metamorphic success, in terms of pigmentation, eye migration and general development, when copepods, rather than enriched *Artemia*, were fed to larvae. Copepods have higher levels of vitamin A, which is required for the synthesis of rhodopsin in the retina, a critical visual pigment in the rods necessary for vision at low light intensities. Deficient rhodopsin affects neural transmission from the retina via the central nervous system that triggers pituitary production of melanophore stimulating hormone leading to reduced melanin synthesis and pigmentation deficiency. DHA, an abundant PUFA in copepods, is also vital to vision as it provides the membrane fluidity necessary for rhodopsin to function when stimulated by light. The essential fatty acids EPA and ArA are more involved in eicosanoid synthesis. These highly potent metabolites are thought to regulate the mechanisms involved in the release of melanophore stimulating hormone and pigmentation. Thyroid hormones play a major role in regulating many developmental processes that occur during metamorphosis. Immersing different age marine fish larvae into various concentrations of thyroid hormone has been shown to synchronize and shorten the duration of metamorphosis in a dose dependent manner in species such as grouper. However, the effect of this immersion on survival varied with the type of thyroid hormone, dose and timing of application. In some species, such as the European seabass (*Dicentrarchus labrax*), females grow up to 40% faster than males. However, when this species is intensively cultured, masculinization can result in a 70-90% male population. A number of studies have shown that manipulating temperature and salinity during larviculture can result in higher quality juveniles, i.e., a higher percent of faster growing females.

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Introduction

The quality of juvenile fish or fry is mainly defined by survival and growth performance during grow-out as well as resistance to stress and disease. The stocking of robust juveniles generally leads to improved fish production, better flesh quality and higher market prices. Juvenile quality is largely dictated by nutritional and environmental conditions during larvae rearing from first feeding to the onset of metamorphosis. Metamorphosis is the transition from larvae to the definitive adult form. During metamorphosis, there are changes in the digestive, respiratory and neural systems as well as in the eye and muscle structure (Jobling, 1995). It is during this time that scales develop, pigmentation strikingly increases and marked changes in behavior are evident. It is becoming increasingly accepted that fish larvae that successfully complete the gamut of physiological and morphological changes occurring during metamorphosis transform into rapidly growing high quality juveniles (Pittman et al., 1998). Consequently, the factors impacting larvae rearing have far reaching implications on much later stages of fish growth and production levels.

Rapidly growing larvae have greater metamorphic success

It has been widely observed that rapidly growing marine larvae generally metamorphose earlier, faster and more successfully than slower growing and less robust cohorts (Næss and Lie, 1998). Larvae that have a higher growth rate characteristically have elevated total lipid levels prior to transformation (Youson, 1988; Koven et al., 1990; Kao et al., 1997; Pfeiler, 1999,). As the processes occurring during metamorphosis exact a high energetic price, fish that are able to rapidly mobilize significant stores of lipid have an advantage in that they can more effectively satisfy maintenance, growth and developmental requirements (Fernández-Díaz et al., 2001) than slower growing individuals. Slow-growing larvae frequently have lower total lipid levels and, often, high mortality at the end of transformation (Christensen and Korsgaard, 1999).

The essential requirement for dietary highly unsaturated fatty acids (HUFA) of the n-3 series, primarily docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), for larval growth and survival in commercial marine species is well documented (Watanabe, 1982; Izquierdo, 1996; Sargent et al., 1999). The phospholipid form of these fatty acids, particularly DHA, plays a critical structural role in enhancing the function of biomembranes in the cells of most tissues. In gilthead seabream, Koven et al. (1990) demonstrated a direct correlation between dietary n-3 HUFA, their selective incorporation into tissue phospholipids and weight gain in larvae (Fig. 1a,b). This was coupled with an effect of essential fatty acids on the total lipid level (Fig. 1c), which likely facilitated the transformation to the juvenile stage. Dhert et al. (1990) found high mortality during metamorphosis in seabass (*Lates calcarifer*) larvae fed diets deficient in EPA and DHA while metamorphosis was accelerated in fish reared on EPA and DHA rich diets offered up to 20 days after hatching. Similarly, a dietary deficiency or absence of DHA or EPA resulted in high mortality, abnormal development and incomplete metamorphosis in turbot (Estévez and Kanazawa, 1995).

Although a direct correlation between the beneficial effects of dietary phospholipids and successful metamorphosis has not been clearly shown, phospholipid supplementation clearly promotes growth and survival during the larvae and juvenile stages of marine fish (Coutteau et al., 1997). Recent studies (Fontagné et al., 1998; Hadas et al., 2003) indicate that dietary phosphatidylcholine, the main phospholipid, improves the mobilization and transport of digested fats from the enterocytes of the digestive tract to body tissues, probably by enhancing lipoprotein synthesis. Apart from contributing to energy accumulation, increasing dietary phospholipids would also increase the availability of the essential fatty acids EPA, DHA and ArA.

Good larvae growth and survival leading to metamorphic success can also be a consequence of beneficial environmental conditions during larvae rearing. A case in point was illustrated with gilthead seabream larvae by

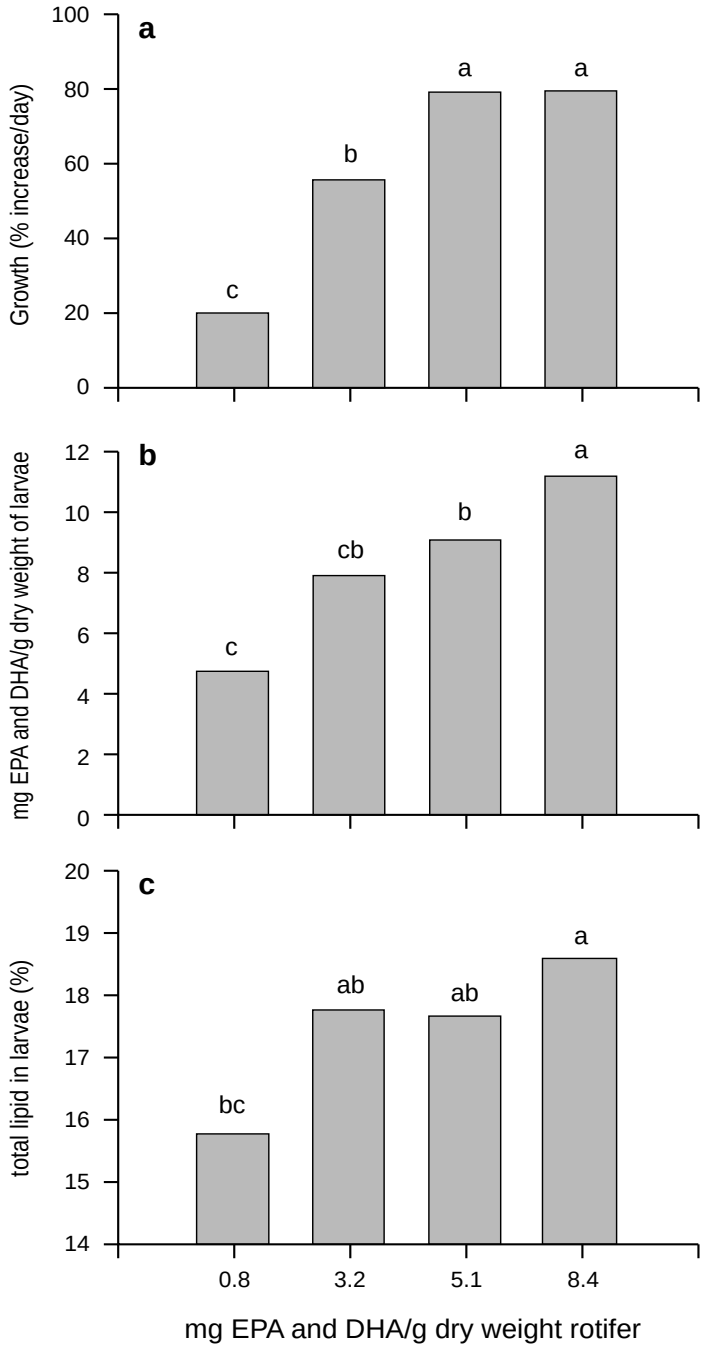

Fig. 1. The effects of different levels of rotifer n-3 HUFA (EPA and DHA) on (a) growth, (b) tissue phospholipids of EPA and DHA and (c) total lipids in gilthead seabream larvae. Treatments with different letters are significantly different (p<0.05). Modified from Koven et al., 1990.

Tandler et al. (1995) who found that reducing the rearing salinity in the ambient Red Sea water (40‰) to 25‰ significantly improved survival, growth and the incidence of swim bladder inflation (Fig. 2a,b,c). Similarly, Specker et al. (1999), working on metamorphosis in summer flounder, found that larvae grown at 8‰ grew better and developed faster than fish reared in 30 and 38‰. In retrospect, perhaps these results are not surprising since in both cases such larvae are found naturally in estuarine areas with characteristically low salinities. Larvae exposed to rearing salinities higher than their natural environment would be metabolically strained by the increased energetic costs of osmoregulation that would subsequently reduce their growth and survival potential and ultimately their metamorphic success.

The effect of live food type on metamorphosis

The most striking examples of the profound morphological and physiological changes that take place during metamorphosis are in flatfish. Larvae transform from symmetric pelagic swimming individuals into asymmetric benthic fish after undergoing a 90º rotation of the body and one-sided pigmentation. The fish lie on the unpigmented side while the eye from this flank migrates to the opposite pigmented ocular side. Poorly pigmented or albino fish, a conspicuous anomaly following incomplete metamorphosis, have little market value and cause significant losses to the industry (Estévez and Kanazawa, 1995) even though the growth rate of malpigmented flat fish appears to be unaffected (Seikai and Sinoda, 1981; Heap and Thorpe, 1987; Seikai et al., 1987).

Studies on Atlantic halibut, turbot and Japanese flounder have reported greater metamorphic success in terms of pigmentation, eye migration and general development when pre-metamorphosing larvae were fed natural marine zooplankton comprised mostly of copepods than enriched *Artemia* (Seikai, 1985; McEvoy et al., 1998; Pittman et al., 1998; Shields et al., 1999). Not surprisingly, natural marine zooplankton are universally regarded as a superior food than *Artemia* nauplii, as they are a richer source of essential fatty acids (Fig. 3), phospholipids, carotenoids, free amino acids and inorganic compounds such as iodine. However, no one has yet developed an industrial protocol for growing natural zooplankton such as copepods in sufficiently high quantities to meet the demands of commercial mariculture. This means that *Artemia* nauplii, despite its nutritional limitations, remain the standard live feed for larvae worldwide primarily due to the availability of *Artemia* cysts on the market and established procedures for hatching and enriching the nauplii with essential fatty acids.

There appears to be a critical window of opportunity, which may be species specific, for feeding copepods to larvae to positively influence metamorphic success. In Atlantic halibut, *Artemia* and copepods served equally well as growth promoters for the first 46 days of feeding (Harboe et al., 1998; McEvoy et al., 1998; Shields et al., 1999). However, after 46 days, larvae that continued feeding on *Artemia* began to show differences in eye migration and pigmentation (Næss et al., 1995) while those that had fed on copepods transformed normally. Næss and Lie (1998) claimed that the copepod window for halibut was 2 to 3 weeks after the first feeding while normally pigmented halibut can be obtained when copepods were fed for only 7 days before larvae reach the myotome height of 2.5 mm associated with eye migration at the initial stage of metamorphosis. The critical period for Japanese flounder was around 15 days after hatching or about 7.5 mm in total length (Seikai et al., 1987) while the pigmentation window for larvae of the yellowtail flounder was up to 9 mm in total length (Copeman et al., 2002). In plaice, on the other hand, the duration of feeding natural zooplankton rather than the exact timing appears to be the relevant factor (Dickey-Collas, 1993). These findings suggest a nutritional factor found in copepods and not in *Artemia* that initiates normal metamorphosis. Researchers initially focused on the essential fatty acids EPA and DHA as well as vitamin A (Estévez and Kanazawa, 1995). Although *Artemia* nauplii are enriched

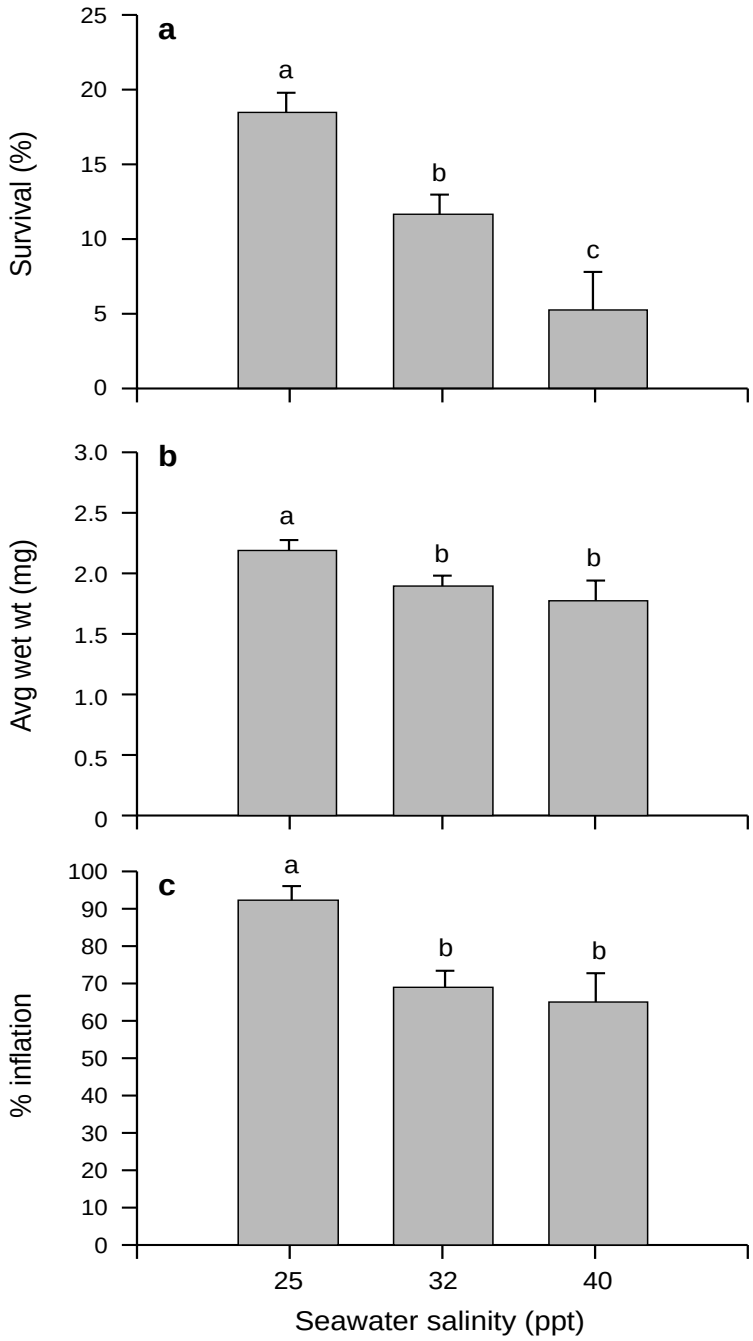

Fig. 2. The effect of reducing salinity on (a) survival, (b) average wet weight and (c) percent swim bladder inflation in gilthead seabream larvae, 32 days after hatching. Treatments with different letters significantly differed (p<0.05). Modified from Tandler et al., 1995.

with n-3 HUFA before being fed to fish larvae, the amounts and ratios of these fatty acids that occur naturally in copepods may be a more favorable trigger for the onset of metamorphosis.

The effect of diet on physiological and morphological processes during metamorphosis

A number of studies (Miki et al., 1989, 1990; Kanazawa, 1991,1993) on flatfish report that the enrichment of *Artemia* with markedly high levels of vitamin A prevented albinism and generally improved pigmentation, although there was a tendency of excessive amounts of vitamin A to produce skeletal deformities (Estévez and Kanazawa, 1995). In fact, immersing Japanese flounder larvae in retinoic acid just prior to the onset of metamorphosis stimulated the formation of adult type chromatophores on both sides of the fish (Miwa and Yamano, 1999). It was also observed that dietary vitamin A supplementation corresponded with retinal development in Japanese flounder (Seikai et al., 1987; Seikai, 1992; Kanazawa, 1993) and that high rates of albinism were correlated with retinal abnormalities. Vitamin A is required for the

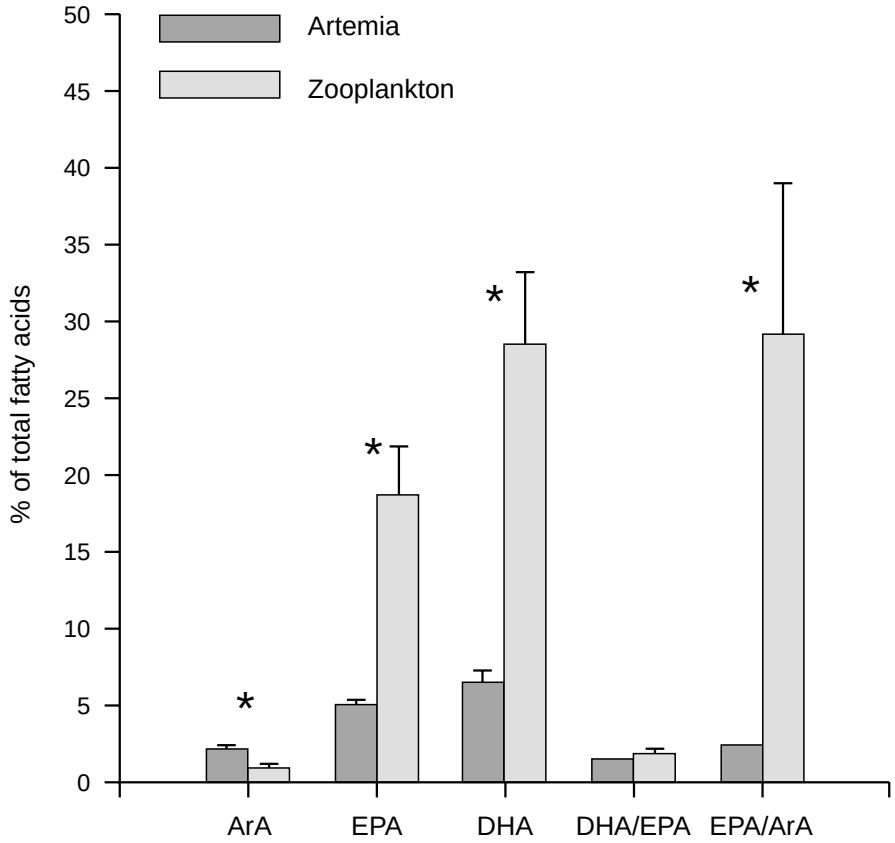

Fig. 3. Typical arachidonic acid (ArA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents and DHA/EPA and EPA/ArA ratios in *Artemia* and natural marine zooplankton fed to Atlantic halibut. Pairs marked with an asterisk significantly differed (p<0.05). Modified from Hamre et al., 2002.

synthesis of rhodopsin in the retina, a critical visual pigment in the rods necessary for vision at low light intensities. Halibut larvae ingesting *Artemia* had considerably fewer rod cells than those feeding on copepods (Fig. 4; Shields et al., 1999). In support of this, Estévez et al. (1997) found that the abnormal retinal epithelium of malpigmented Japanese flounder was nutritionally induced and resulted in visual deficiency. If rhodopsin synthesis is interrupted by deficient vitamin A, this could in turn affect the neural transmission from the retina via the central nervous system that triggers pituitary production of melanophore stimulating hormone leading to reduced melanin synthesis and pigmentation deficiency.

Membrane fluidity of the photoreceptor rod cells can effect rapid conformational changes that occur in rhodopsin once it is stimulated by photon absorption. These changes are necessary to initiate a cascade of events culminating in the hyperpolarization of neural membranes and the propagation of a signal down the central nervous system that stimulates the pituitary to produce melanophore stimulating hormone. Tissue phospholipid DHA is associated with membrane fluidity due to the unique

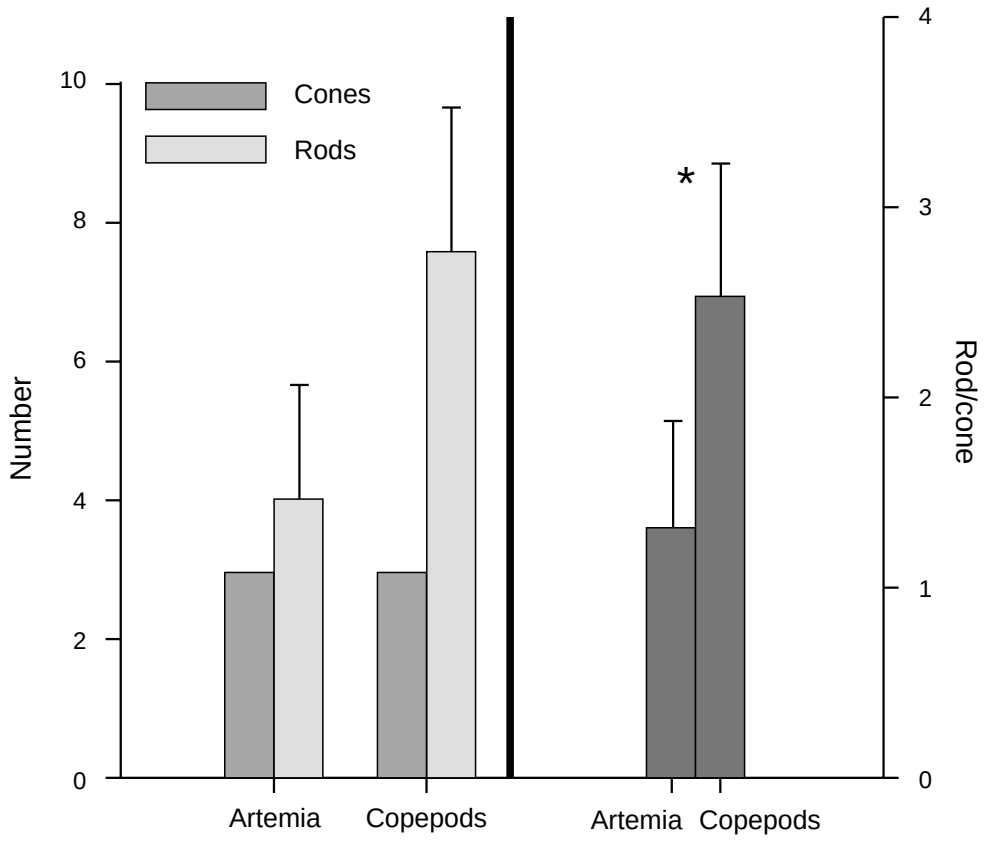

Fig. 4. The number of rods and cones and rod/cone ratios in retinas of halibut larvae fed enriched *Artemia* or *Eurytemora velox* copepods. The rod/cone ratio in larvae fed copepods was significantly higher (p<0.01) than in larvae fed *Artemia*. Modified from Shields et al., 1999.

structural configuration of this fatty acid and is found in high concentrations in photoreceptors of the retinal pigment epithelium (Bell and Dick, 1993). Estévez and Kanazawa (1996) found that the brain and eyes of poorly pigmented fish had less phospholipid DHA than those of normally pigmented individuals. Vitamin A, DHA and phospholipids have all been deemed essential for the promotion of normal pigmentation (Miki et al., 1990; Dickey-Collas, 1993; Reitan et al., 1994) and all three are found in high concentrations in copepods (Estévez and Kanazawa, 1995, McEvoy et al., 1998; Shields et al., 1999).

As DHA has a more dominant role in growth (Watanabe, 1993) and membrane structure than EPA, a number of authors reported that a high dietary DHA/EPA ratio was frequently associated with good pigmentation (Rainuzzo, 1993; Reitan et al., 1994). Reitan et al. (1994) suggested that a dietary DHA/EPA ratio of 1.7 would result in 90% pigmentation. However, there are inconsistencies in the literature regarding the efficacy of dietary DHA/EPA as a true index of expected pigmentation success (Estévez and Kanazawa, 1996; Copeman et al., 2002). Estévez and Kanazawa (1995) found a high correlation coefficient between the DHA/EPA ratio in *Artemia* and successful turbot pigmentation but noted a poor association between this parameter and the DHA/EPA ratio in turbot fry. In a more recent study, Estévez et al. (1999) found no relationship between DHA/EPA in rotifers, *Artemia* or the brain and pigmentation rate in turbot and, in fact, found that fish fed rotifers and *Artemia* with low DHA/EPA ratios had pigmentation rates higher than 80%. Similarly, McEvoy et al. (1998) found no significant difference in DHA/EPA ratios between tissues from normal and malpigmented halibut fry. Hamre et al. (2002) reported that Atlantic halibut fed copepods containing elevated DHA levels had a higher incidence of normal pigmentation than fish fed *Artemia* with a lower level of DHA, despite the fact that the DHA/EPA ratios in these zooplanktons are very similar. A number of workers concurred that absolute levels of DHA and arachidonic acid (ArA, 20:4n-6) have more biological relevance to pigmentation success and are more selectively assimilated into neural tissue than EPA (Næss and Lie, 1998; Hamre et al., 2002). Consequently, studies generally now focus on the DHA/ArA and EPA/ArA ratios in tissues.

In contrast to DHA, the fatty acids EPA and ArA play a minor role in membrane function and are more involved in eicosanoid synthesis. Eicosanoids represent a broad range of highly biologically active metabolites that include mainly prostaglandins, thromboxanes and leukotrienes. These hormones are involved in various areas of cellular regulation including control of fluid and electrolyte fluxes, the cardiovascular system, reproductive function, the neural system (Mustafa and Srivastava, 1989; Bell et al., 1994) and metamorphosis. However, ArA and EPA compete for binding to cyclooxygenase enzymes that synthesize eicosanoids such as prostaglandins. ArA is the preferred substrate for eicosanoid production and 2-series prostaglandins derived from ArA (PGE_2) are more biologically active than the 3-series synthesized from EPA (PGE_3; Sargent et al., 1999). This means that PGE_3 appears to have an important role regulating levels of the more potent PGE_2 metabolites.

During metamorphosis in flatfish, ArA eicosanoids stimulate the synthesis of cAMP and cGMP in the brain and eyes (Estévez and Kanazawa, 1996). The second messenger cAMP signals the dispersion of pigment while cGMP binds to Na+ channels in the outer segment membrane of rods causing these channels to remain open. When rhodopsin in the rods is light activated, a series of enzymatic events occur which converts cGMP to 5'GMP. Consequently, the cGMP intracellular concentration drops, resulting in the closing of the Na+ channels. This causes the rod photoreceptors to become hyperpolarized, which propagates a central nervous system signal that stimulates pituitary melanophore stimulating hormone and pigmentation (Estévez and Kanazawa, 1996). If cGMP levels are regulated by ArA derived eicosanoids, then different tissue EPA/ArA ratios would affect the available lev-

els of cGMP and subsequently the neural and neural endocrinological control of metamorphosis and pigmentation success.

The effective tissue EPA/ArA ratio signaling normal release of melanophore stimulating hormone and pigmentation may be species and developmental stage specific. Estévez et al. (1997) found that increasing ArA produced higher growth and pigmentation success in Japanese flounder juveniles after 45 days of feeding. Sargent et al. (1999) proposed that striped bass might require an EPA/ArA ratio of less than 1:3 during metamorphosis and seabass a ratio of 1:1. On the other hand, significant correlations were found between the ArA concentration in turbot and halibut brains and increased incidence of malpigmentation (McEvoy et al., 1998; Estévez et al., 1999) where the greatest number of normally pigmented fry occurred when the brain EPA/ArA ratio was over 4:1.

Dietary influence on cortisol and thyroxine hormones during metamorphosis

Recent studies on gilthead seabream suggest that increased levels of dietary ArA down-regulated cortisol levels during acute or handling stress and up-regulated basal cortisol during chronic stress (Koven et al., 2001, 2003). Cortisol not only restores the hydromineral balance but also catabolizes energy yielding substrates during stress responses. This means that dietary ArA may also modulate the characteristically high cortisol peak observed in fish larvae at the onset of metamorphosis (Tanaka et al., 1995). In this case, cortisol likely mobilizes lipid reserves that, as previously mentioned, are required to meet the considerable energy demands of this developmental stage. As metamorphosis is a process lasting weeks in some species, high cortisol levels may be a response to the chronic stress of morphological and physiological changes, suggesting that higher dietary ArA levels at this time may be required.

The high cortisol peak during early metamorphosis in fish is followed by increased levels of thyroid hormones (TH), thyroxine (T_4) and triiodothyronine (T_3; Tanaka et al., 1995). TH have a major role in regulating many of the developmental processes that occur during metamorphosis such as gastric development in Japanese flounder (Miwa et al., 1992) and eye migration in halibut (Solbakken et al., 2002). However, cortisol and TH appear to act synergistically on many developmental processes such as fin reabsorption in flounder (de Jesus et al., 1990).

Since Lam (1980) first reported the positive effect of T_4 treatment on larval development and survival of tilapia, numerous trials using exogenous TH have been conducted with the aim of developing practical protocols. Brown et al. (1988, 1989) showed that survival of striped bass larvae could be improved by supplementing TH in the eggs via maternal injections of a high dose of T_3 a few hours prior to spawning. In a later study, improved survival of Hawaiian threadfin larvae was observed after immersion of newly fertilized eggs in solutions of T_3 and combinations of T_3 and cortisol (Brown and Kim, 1995). However, similar treatment with T_4 in the rabbitfish had no effect (Ayson and Lam, 1993). Recent publications reported that immersing pre-metamorphosing grouper (*Epinephelus coiodes*) in various concentrations of T_4 and T_3 resulted in the synchronization and shortening of the duration of metamorphosis in a dose dependent manner, independent of larval size (de Jesus et al., 1998). Their TH doses were comparable to those inducing metamorphosis in flounder larvae (Inui and Miwa, 1985; Miwa and Inui, 1987) and the appearance of black stripes and settlement in the red sea bream (Hirata et al., 1989). The accelerated onset of pigmentation and differences in swimming behavior among TH-treated larvae suggest an influence of this hormone on the peripheral development of the neuromuscular system (Brown and Kim, 1995). Yamano et al. (1991) showed that T_4 treatment accelerated the shift from larval to juvenile type muscle proteins in the Japanese flounder, coincident with the onset of benthic behavior. Reabsorption of the dorsal fin rays of grouper larvae was also advanced by TH treatment. The effect of T_3 was more pronounced than

that of T_4 in all age groups but was dependent on the dose. Similar observations were reported for flounder (Miwa and Inui, 1987; de Jesus et al., 1990). Most reports show that TH improve survival rates and accelerate growth and development in teleost larvae (Brown and Bern, 1989; Lam, 1994). On the other hand, de Jesus et al. (1998) reported that the effect of TH immersion on survival varied with the type, dose and timing of application of the TH. Old et al. (1992) reported that T_3-induced precocious expression of thyroid hormone receptor in *Xenopus* embryos resulted in the development of abnormalities. Deleterious effects of early TH treatment were found in the larvae of striped bass (Huang et al., 1996) and seabass (Nugegoda et al., 1994). The emerging picture from these studies is that hormone immersion is an effective protocol causing synchronization and shortening of metamorphic development and enhancement of juvenile quality in commercially valuable fish although this approach may prove costly in large-scale operations and raise justifiable questions among an increasingly environmentally aware public.

Arguably, a better approach to stimulating TH synthesis in pre-metamorphosing larvae is through the diet. Another reason why larvae fed natural zooplankton such as copepods metamorphose more successfully is that copepods contain up to 700 times more iodine, the major precursor of TH synthesis, than *Artemia* nauplii (Fig. 5; Solbakken et al., 2002). On the other hand, *Artemia* and copepods have similar levels of tyrosine (an amino acid precursor of TH) and selenium (necessary for the conversion of T_4 to T_3) and a small though significant difference in the TH amino acid precursor, phenylalanine (Solbakken et al. , 2002).

Improving juvenile quality by controlling sex differentiation during larval development

One of the characteristics of the commercially grown European seabass (*Dicentrarchus labrax*) is that females can grow up to 40% faster than males. This means that producing female dominant populations would represent a significant increase in production biomass and profitability. However, when this species is grown in intensive culture, the fish typically exhibit massive masculinization where 70-90% of the population can be males. Studies carried out at our laboratory in Eilat found that exposing larvae to lower temperatures during the first 50 days after hatching or juveniles 50-100 days after hatching had, at a much later stage of development, a significantly higher incidence of females. Pavlidis et al. (2000) similarly found that 13-15ºC during very early developmental stages affected sex differentiation in seabass, resulting in a consistently higher proportion of females. These studies emphasize that higher quality juveniles, in terms of a higher percent of faster growing females, can be attained by temperature manipulation during larviculture.

In nature, larvae and juvenile seabass grow in temperate estuaries (Kelly, 1988) or lagoons (Barnabé, 1976) and can be exposed to salinities of 10-20‰. Saillant et al. (2003) found that when seabass of 44 mm were transferred from low to high salinity, the percentage of males in the population increased. Poor larval performance in higher salinities may be linked to a limited osmoregulation capacity that improves in older fish (Tandler et al., 1995; Harel et al., 1998). Saillant et al. (2003) suggested that osmotic stress and possibly higher cortisol levels during a critical stage of fish development may mobilize energy reserves away from the development of large nutrient-rich ovaries and into the less energy demanding growth of male gonads. These authors also claimed that long-term growth of seabass juveniles is not influenced by salinity when temperature is high (ca 22ºC), indicating that the rearing temperature may be a more dominant factor influencing energy allocation than salinity and supporting recent studies at our institute. These results suggest that both low temperature and low salinity may increase the incidence of females in a population.

In summary, environmental (e.g., temperature, salinity) and nutritional (e.g., DHA, EPA, ArA, vitamin A, phospholipids, iodine) factors during larvae rearing determine the degree of successful transformation from

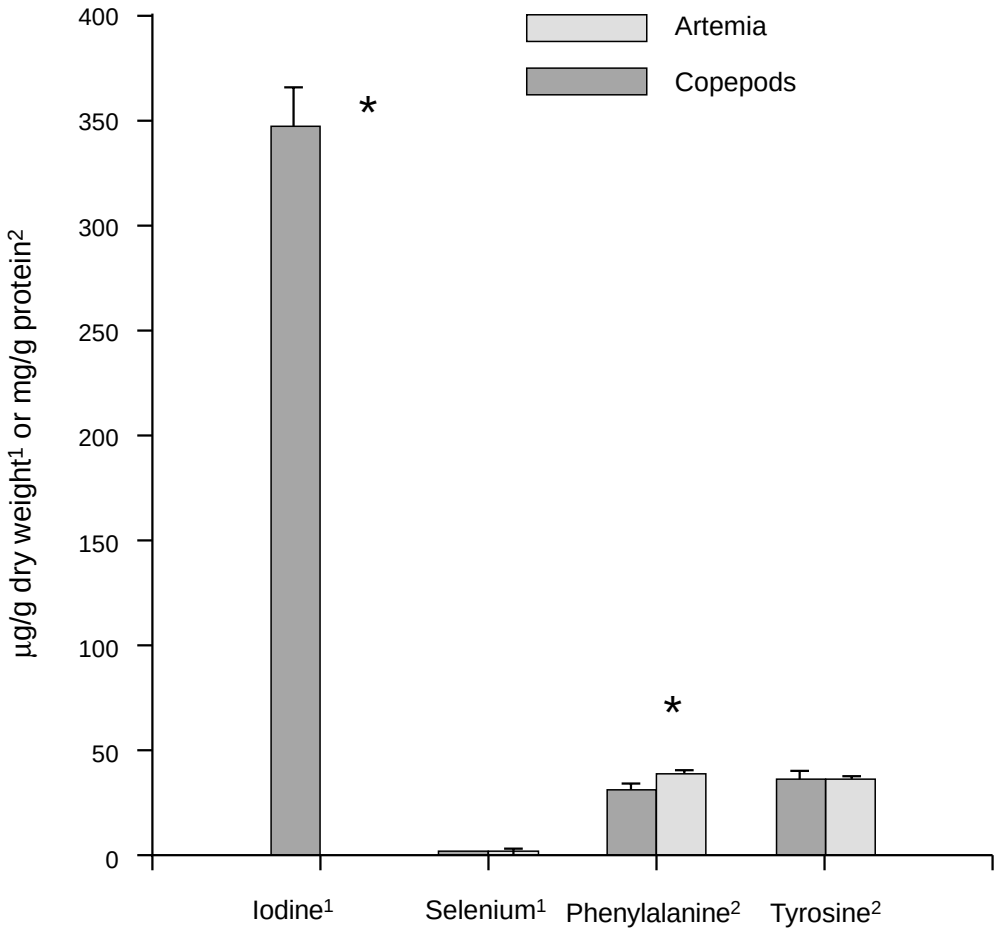

Fig. 5. Iodine, selenium and free amino acid concentrations in wild zooplankton and *Artemia*. Iodine values significantly differed at the $p<0.00001$ level while phenylalanine values significantly differed at the $p<0.05$ level. Modified from Solbakken et al., 2002.

larva to juvenile during metamorphosis. Metamorphic success is the main element influencing juvenile quality which, in turn, affects the cost and level of fish production at the end of the grow-out period.

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